In planta assimilation and characterization of metal nanoparticles in *in vitro* shoots of *Withania somnifera* and its therapeutic evaluation using Rotenone induced SH-SY5Y cells

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Plants have rich source of primary and secondary metabolites and all living organisms have been nutritionally depended on them directly or indirectly. Medicinal plants are capable of producing unique bioactive compounds that are specific to species or family of plants. The evolution of secondary metabolism in plants is astonishing event which created thousands of bioactive compounds with unimaginable power to restore health in a human being. Researchers all around the world trying to understand the vast application of secondary metabolites to cure diseases and disorders. Of course, we are not even half way through the looking glass. We still need to put enormous efforts to understand the plant kingdom and its components. The present study adds a single step to this long walk ahead of us.

Here, a medicinal and ayurvedic plant, *Withania somnifera*, is taken into study its reduction and therapeutic potential under *in vitro* condition. As any other medicinal plant, *W. somnifera* has a unique secondary metabolite composition including Withanolides. Withanolides are steroidal lactones belongs to the group of triterpenoids with enormous therapeutic potential. It was known to have anti-cancer, anti-diabetic, anti-inflammatory, neuro-protective activity among others. There are more than 40 withanolides has been reported until today and among them withaferin A, withanolides A and withanone is most studied and has increased therapeutics. Field grown *W. somnifera* tissues are used in more than 200 traditional formulations. Due to industrialization and urbanization, heavy metals and pesticides level along with other toxic compound level is increased in soil which eventually transported into the food chain via food crops and medicinal plants. Further, *W. somnifera* has a hyperaccumulation capability that increases the percentage of heavy metal accumulation compared to non-accumulator plant species. Thus, the field grown *W. somnifera* tissues that are grown near contaminated area is not suitable for medicinal purposes. Further, field grown plants tend to have seasonal and geographical variations which their metabolites content which makes them batch to batch variations in their final product. Thus, *in*

vitro tissue cultures overcome these metabolite variations due to environmental stressor, heavy metal contamination or other adulteration.

Taking metal accumulator capability of *W. somnifera* into consideration, *in vitro* shoot cultures were selected for metal toxicity studies. Two heavy metals were selected Pb and Ag; Pb was selected for its heavy metal and lower solubility and immobile in soil and Ag for its high reactivity, easily reducible and useful as a therapeutic agent. Metal toxicity studies were done on suspension (liquid) medium with hormone (BAP) supplementation to increase the free availability of metal ions to shoot cultures and BAP used to increased the uptake and translocation of metal ions by shoots.

Thus, the present study on **"*In planta* assimilation and characterization of metal nanoparticles in *in vitro* shoots of *Withania somnifera* and its therapeutic evaluation using Rotenone induced SH-SY5Y cells"** is formulated with the following objectives

1. To study the effect of heavy metal salts on *Withania somnifera* shoot growth and withanolides production.
2. To quantify and analyse the nature of bioaccumulated metal ions in *in vitro* grown shoots.
3. Evaluating the neuroprotective activity *W. somnifera* tissues in Parkinson's' disease cell model.

The study was carried out in three phases. In the first phase, the metal reduction capability of field grown shoots of *W. somnifera* was analysed using two metal salts: Silver nitrate ($AgNO_3$) and lead acetate (PbAc) solution. The green synthesis conditions were optimized and physical and chemical characterization was done to confirm the green synthesis of Ag NPs, and Pb NPs. In addition, metal bioaccumulation capability of field grown shoot and root of *W. somnifera* grown in non-contaminated soil was also studied. Once the reduction potential and bioaccumulation capability of field grown shoots of *W. somnifera* was determined, further studies were carried out using *in vitro* shoot. In the second phase, standardization of metal treatment using two abiotic stressors: $AgNO_3$ and PbAc were studied for improved growth index and its influence on withanolides

accumulation. The metal salts were treated under two different treatment conditions such as acute toxicity studied and chronic toxicity studies. Acute toxicity studies, high concentrations of metal salts were treated for shorter period which determines the threshold of *in vitro* shoot system for a specific metal treatment. Chronic toxicity studies, low concentrations of metal salts were treated for longer period which is to understand the shoots' ability to withstand metal treatment over a long duration. Chronic toxicity studies were usually conducted for field experiments because long duration and low concentration facilitates the plant to adapt to the stressors which may results in different observation that of acute studies. Acute and chronic metal toxicity studies on *in vitro* shoot resulted in increased growth index and Withanolide production. Healthy shoot cultures with increased GI and withanolides production was selected as the optimum culture conditions for each metal. This study also confers Ag and Pb bioaccumulation at optimum exposure condition. The ultra-structural studies were done to identify the nature of bioaccumulated metal in metal treated *in vitro* shoots of *W. somnifera*. The third phase includes a therapeutic efficiency of optimum concentration of silver nitrate treated *in vitro* shoots along with field tissues of *W. somnifera* and withaferin A were studied using rotenone induced Pakinson's disease model in SH-SY5Y cell lines. The induction of cell viability by *W. somnifera* extract pre-treatment against rotenone induced cell toxicity was studied. The percentage of ROS accumulation, apoptotic cells and mitochondrial membrane potential in rotenone treated SH-SY5Y cells and *W. somnifera* extracts pretreated rotenone treated SH-SY5Y cells was also analysed. Mitochondrial gene expression study was done to analyse the effect of pre-treatment of *W. somnifera* extracts on rotenone treated SH-SY5Y cells. The interaction of rotenone and three important withanolides with electron transport chain (ETC) complex I protein was studied using molecular docking analysis.

The metal bioaccumulation and reduction capability of field grown shoots (FGS) of *W. somnifera* is found to be increased compared to field grown root (FGR). Initially, to identify the metal bioaccumulation capability of field grown tissues of *W. somnifera*, the field grown plants from non-contaminated were harvested, dried and nitric acid digested tissues were taken for elemental

analysis. The elemental analysis includes essential trace metals and essential heavy metals for plant growth and non-essential heavy metals for plant growth with totalling of 22 elements. Compared to FGR, FGS accumulated increased amount of both trace and heavy metals for plant growth which is normal considering that shoot is the major site for photosynthesis, metabolites production and accumulation. In addition, all trace and heavy metals from FGS and FGR found within permissible set for medicinal plant and edible plant. The presence of non-essential heavy metals is found to be increased in FGR for Cr, Co, As, and Hg content compared to FGS. On the other hand, increased amount of Cd, Ag, Pb, V and Li was found in FGS compared to FGR. Further, the selected non-essential heavy metals for plant growth Ag and Pb, both are found slightly higher in FGS compared to FGR. Therefore, due to the increased metal bioaccumulation capability of FGS, metal reduction studies were conducted using FGS alone. Green synthesis of metal nanoparticles was conducted using two heavy metal salts (AgNO3 & PbAc), two experimental conditions (Room temperature and 60ºC (Hot plant)) and FGS extracts. The green synthesis of Ag NPs was found to be positive for both experimental conditions. However, Ag NPs synthesized at 60ºC is found to be less stable after 2 days of experiment which was confirmed using UV-visible spectrophotometry analysis. Thus, Ag NPs synthesized at room temperature is found to be stable for 30 days using UV-vis spectrophotometry and taken for other physical and chemical characterization studies. Green synthesis of Pb NPs using both experimental conditions were failed. FESEM analysis on Ag NPs were found that the synthesized nanoparticles were in cubic shape with size range from 20-80 nm. EDAX analysis confirms that 67% percentage of Ag was present in the analysed samples. FTIR spectrum shows the surface molecules of Ag NPs includes phenols, terpenes and protein molecules which in fact proves that plant metabolites were responsible for the reduction and stabilization of Ag NPs.

Thus, the present study was formulated based on *W. somnifera*'s increased therapeutic and reduction potential. Two non-essential heavy metals such as silver and lead was selected. The metal salts were treated with *in vitro* shoot cultures and the concentration of metal salts were selected based on Indian

contaminated and non-contaminated soil metal concentration from the available literature reports.

The influence of metal toxicity studies in the second phase revealed that both metals did not exhibit any toxicity symptoms at low concentration treatment to longer period on IVS cultures. On the other hand, high concentration of metal treatment to shorter period exhibited toxic symptoms in IVS at higher concentrations. Among acute toxicity studies, E1H3T3 produced 1.8-fold increase in WFA content (1.097±0.01e mg/g DW) compared to control (CT3) but high concentration of silver treatment negatively correlated with GI at all concentration and durations. Further, E2H3T3 produced 1.6 and 1.97 fold increase in GI (0.1772±0.01c) and WFA content (1.184±0.01c mg/g DW) respectively compared to control (CT3). Whereas on chronic toxicity studies, E1D3T6 showed maximum GI (1.425±0.05c) and WFA content (2.568±0.08e mg/g DW) which was 4.5 and 3.4 fold greater than the control (CT6). On the other hand, on Pb treatment, E2D4T6 showed maximum GI (2.211±0.04d) which was 7 fold greater than the control whereas E2D5T6 showed maximum WFA (3.137±0.01f) which was 4.1 fold greater than the control. Thus, among all acute and chronic metal toxicity studies, E1D3T6 and E2D5T6 showed best elicitation activity and taken as optimum culture conditions for Silver and Lead, respectively.

Following that the HPTLC profiling of withanolides in E1D3T6, E2D5T6, control (CT6) shoots was performed in comparison to FGS and FGR against standard WFA, WTA and WN. Compared to control shoots (0.783 mg/g DW), all the other tested samples has increased amount of WFA except FGR (0.608 mg/g DW). Maximum WFA content was observed in FGS (6.607 mg/g DW) following E2D5T6 (3.120 mg/g DW) and E1D3T6 (2.544 mg/g DW). There was no significant difference was observed in WTA content in all the analysed samples except that FGS (0.142 mg/g DW) had minor quantity of WTA compared to other tested samples. Compared to control (0.805 mg/g DW), WTA in E1D3T6 (0.600 mg/g DW) was decreased but in E2D5T6 (0.960 mg/g DW) increased. A maximum WN content was found in FGS (2.285 mg/g DW) following FGR (1.707 mg/g DW) and control (1.385 mg/g DW). Compared to control, WN content in both E1D3T6 (0.709 mg/g DW) and E2D5T6 (1.027 mg/g DW) was decreased significantly.

Therefore, withanolides quantification on metal treated and control samples revealed that metal treatment positively correlated with WFA and WTA content and negatively with WN content compared to control, except for E1D3T6, WTA level decreased.

Bioaccumulated metal concentration in E1D3T6 and E2D5T6 was analysed using ICPMS. In elemental analysis, eight essential trace metals, four essential heavy metals and ten non-essential heavy metals were quantified in E1D3T6, E2D5T6 and control (CT6) shoots. The Ag bioaccumulation in E1D3T6 was quantified as 50.8 ppm and Pb bioaccumulation in E2D5T6 was quantified as 405 ppm. Despite higher Pb concentration in their tissues, the shoots were in good shape and healthy. As expected, control was void of both Ag and Pb. Moreover, the percentage of Ag in E1D3T6 shoots was quantified as 2.54% and Pb in E2D5T6 was quantified as 15.14%.

The presence of increased heavy metal tolerance in *in vitro* shoot cultures of *W. somnifera* is observed. Resistance to the toxicity of heavy metal accumulation is observed with increase in macronutrients such as K, Ca and Fe in E1D3T6 and E2D5T6 compared to control. These increased macronutrients are reported to protect plants from biotic and abiotic toxicity. The concentration of K is increased to 26077.9 (E1D3T6) and 24779.14 mg kg^{-1} (E2D5T6) from 21109.36 mg kg^{-1} (control), Ca level is increased to 1833.64 (E1D3T6) and 1786.93 mg kg^{-1} (E2D5T6) from 1411.68 mg kg^{-1} (control), similarly, Fe level is increased to 85.101 (E1D3T6) and 82.1 mg kg^{-1} (E2D5T6) from 11.2 mg kg^{-1} (control) during heavy metals stress.

To analyse the nature of bioaccumulated silver and lead in E1D3T6 and E2D5T6 shoots, ultra-structural analysis using transmission electron microscopy. Initially, E2D5T6 shoot was analysed for the presence of possible nanostructures. It was found that bioaccumulated Pb ions (405 mg kg^{-1}) was in fact in the form of nanostructures in the shape of spherical and rod and the size ranging from 25-40nm. Further, many reports suggest that that the absorbed metal gets reduced at the root via root cell wall polysaccharides/organic acids or during translocation (root to shoot) in apoplasts/symplasts or at the greener regions (shoots and

leaves). The translocation of heavy metal ions in the plants is mainly by apoplastic and symplastic pathway which was detailed in the phase two results. Even though exact mechanism of metal nanoparticles synthesis and accumulation is unknown, many reports suggests that the phytoconstituents present in *W. somnifera* shoots might have acted as both a reducing and stabilizing agent, which results in the formation and accumulation of Pb NPs. The accumulation of increased amount of Pb shows that *W. somnifera* shoots have advanced metal detoxification and sequestration system. Therefore, increased withanolides content, growth index, improved metal detoxification system and increased metal translocation coefficient of *in vitro* shoot of *W. somnifera* resulted in the synthesis of accumulation of Pb NPs. In addition, this is the first report on *in planta* synthesis and accumulation of Pb NPs.

Similar result was observed in E1D3T6 where synthesis and accumulation of Ag NPs was confirmed using electron microscopy and EDX analysis. In E1D3T6, Ag NPs found in spherical and rod shaped and the size ranging from 20-90nm. Ag absorption, translocation and accumulation is much similar to Pb ion due to their similar ionic characteristics. The presence of increased secondary and primary metabolite content also increases the reduction and stabilization of Ag NPs at *in planta* level. There are many other reports showing the *in planta* synthesis of Ag NPs in plants such as *Medicago sativa*, *Brassica juncea*, *Arabidopsis thaliana* and etc. These studies proved that the location of Ag NPs accumulation is in cell wall, plasmalemma and cell organelles such as vacuoles, chloroplast and mitochondria.

The reduction potential of *in vitro* shoots of *W. somnifera* towards Ag and Pb was confirmed. However, for further studies, E1D3T6 shoots i.e., 1mM $AgNO_3$ shoots for 12 days period, containing Ag NPs was taken to analyse its neuroprotective activity in rotenone induced SH-SY5Y cell line. E2D5T6 shoots was not used for therapeutic studies due to their heavy metal nature. Though, Ag is considered as a heavy metal, it has increased pharmaceutical activity which can be useful.

Rotenone is used to induce Parkinson's disease symptoms in SH-SY5Y neuroblastoma cells. Rotenone is a pesticide/ isoflavone which capable of inhibiting mitochondrial electron transport chain complex I protein. Inhibition of complex I resulted in PD symptoms like decreased mitochondrial dysfunction, energy metabolism and increased dopaminergic neuronal loss in the substantia nigra. For neuroprotective studies, E1D3T6, control shoots, FGS, FGR and WFA was selected. Initially, MTT assay was done to analyse the cell viability of selected samples against rotenone induced SH-SY5Y cells. The result shows that FGS exhibited more toxicity and failed to revive the SH-SY5Y cells from rotenone induced toxicity. Further, E1D3T6 (48.28 µg/ml), IVS (43.64µg/ml) and FGR (49.57µg/ml) and WFA (50.05µg/ml) exhibited increased cell reviving ability against rotenone induced toxicity. From these results, we concluded that FGS is more toxic to be used in therapeutic studies hence, FGS along with IVS was removed in further studies.

ROS generation, mitochondrial membrane potential and apoptosis in rotenone treated SH-SY5Y cells was analysed using flowcytometry assay. Reactive oxygen species (ROS) are produced intracellularly at various locations including mitochondrial electron transport chain complexes like complex I and complex III. Increased ROS accumulation results in the damaged biomolecules which in turn acts as potential contributors to a range of diseases including neurodegenerative diseases. ROS scavenging assay was done using H2DCFDA staining. In control cells on treatment of rotenone, 55% ROS accumulation and aggregation was observed. However, pre-treatment with E1D3T6, FR and WFA significantly reduced the ROS generation in SH-SY5Y cells after rotenone treatment to 16%, 36% and 36%, respectively.

Depolarization and hyperpolarization of mitochondrial membrane led to reduction in transmembrane potential which resulted in the activation of mitophagy or autophagy. Mitochondirial membrane potential was anlaysed by JC-1 staining. In rotenone treated control SH-SY5Y cells, percentage of monomer level was increased to 69.74% due to mitochondria depolarization. On the other hand, during *W. somnifera* extracts and WFA pre-treatment, the percentage of aggregates was increased from 29.90% (control cells) to 86.81% (E1D3T6),

78.78% (FGR) and 71.11% (WFA) which suggests that mitochondrial transmembrane potential was reversed back to normal on treatment. Among tested samples, E1D3T6 pre-treated Rot induced cells exhibited increased level of aggregates (86.81%) compared to other samples (FR and WFA) showing high neuroprotective activity.

Apoptosis is a programmed cell death responsible for DA neuronal death in pathophysiology of Parkinson disease. Rotenone treatment causes mitochondrial damage which led to DA neuronal loss (apoptosis). Apoptosis was analysed by Annexin V/ PI binding assay. The percentage of early and late apoptotic cells in rotenone treatment were 47.26% and 37.22% and only 13.27% live cells were quantified. the pre-treatment of E1D3T6, FGR and WFA showed higher percentage of live cells 86.61, 83.17 and 84.89 respectively, however, Rot treated control cells showed only 13.27% live cells. In addition, Rotenone treated SH-SY5Y cells showed 37.22% late apoptotic cells (LAC) and 49.26% early apoptotic cells (EAC) which was reversed to 0.56, 4.09, 3.72% of LAC and 0.05, 11.37, 11.34% of EAC for E1D3T6, FR and WFA pretreatment respectively.

For gene expression studies, two important mitochondrial genes such as PINK1 and DJ-1 was selected along with GAPDH as a housekeeping genes. Primer validation was done using qPCR and SYBR reactions. All primers (PINK1, DJ-1/PARK7 and GAPDH) were amplified the expected size product with no self-annealing or self-dimerization property. In melt curve plot, a single peak was detected for all primer pairs. PCR amplification efficiencies ranged from 90.30 to 97.19%.

PINK1 (PTEN-induced kinase 1), acts together with Parkin in the maintenance of mitochondrial quality control. Mutation in either of these gene leads to oxidative stress mediated aggregation of α-synuclein and mitochondrial dysfunction causing sporadic Parkinson’s disease. The induction of mitochondrial dysfunction and neurodegeneration by rotenone caused 18.5-fold increase in PINK1 expression in rotenone treated SH-SY5Y cells. During pretreatment of E1D3T6, FR and WFA, PINK1 gene fold change was reverted back to 5.7-, 3.5-,

and 10.2-fold from 18.5-fold in rotenone treated SH-SY5Y cells restoring the normal function of neuronal cells.

Another important gene we have selected for the present study is DJ-1. Loss-of-function DJ-1 mutations related to autosomal recessive early-onset Parkinsonism. DJ-1 is an oxidative stress sensor and an antioxidant. Oxidative stress levels in mitochondria of SH-SY5Y cells upregulates DJ-1 expression which plays important roles such as anti-oxidant activity, mitochondrial function and neuroprotection. However, pretreatment of E1D3T6 and FR extracts and WFA of SH-SY5Y cells following rotenone treatment causes restored DJ-1 expression to normalized levels. Compared to untreated cells, DJ-1 expression in rotenone alone treated cells (positive control) is 7.5-fold up regulated. This effect was reversed when the SH-SY5Y cells were pretreated with E1D3T6, FR and WFA following rotenone treatment to 2.02, 5.58 and 3.7-fold respectively. This shows that oxidative stress formed due to rotenone treatment causing up regulation of DJ-1 in SH-SY5Y cells.

The mechanism of rotenone inhibiting ETC complex I chain is reported by many researchers. ETC complex I proteins has 44 protein subunits and the binding site for rotenone in ETC complex I proteins are NDUFS2, NDUFS7, ND1 and ND4. However, the mechanism of withaferin A and other important withanolides such as withanolide A and withanone with ETC complex I chain protein subunits is unknown. Therefore, the present study was extended to analyse the interaction of three withanolides with selected complex I protein subunits using molecular docking analysis. We have selected important complex I protein subunits having rotenone binding site and NADH dehydrogenase enzyme active site. Total seven protein subunits were selected based on the literature survey such as NDUFV1, NDUFS1, NDUFV2, NDUFS2, NDUFS7, ND1 and ND4. Among the selected subunits, all three withanolides and rotenone was found to be bind with same active site having overlapping binding pocket amino acids. Among withaferin A and rotenone, overlapping binding pocket aminoacids that were found is Arg:111, Ser:205, Glu:204 and Met:197. The interaction of these amino acids with the selected ligand is found as conventional hydrogen bond and carbon hydrogen bond. Apart from this, overlapping binding pocket amino acids between

withanolide A and withanone was found such as Pro:89, Leu:192 and Met:197. However due to the presence of alkyl bonding and absence of conventional hydrogen bonding between withanolide A and complex I protein, withanolide A was not selected for multiple ligand docking studies. Among withanone and rotenone, we have also found overlapping binding pocket amino acids such as Arg:111, Glu:204 and Ser:205. Unfortunately, due to the presence of unfavourable acceptor-acceptor bonding withanone was also not selected for the multiple ligand docking studies.

Multiple ligand docking was performed for withaferin A and rotenone with selected protein subunits of complex I protein. The docking results shows that the binding was occurred between withaferin A and rotenone which inhibits the rotenone from binding with complex I protein. The two-ligand binding instead of their binding with receptor molecules indicates withaferin A's competitive inhibition of rotenone. Further, withaferin A and rotenone binding leaves the complex I protein uninhibited. The absence of binding rotenone with the complex I protein results in the normal function of mitochondrial electron transport chain and normal ATP production. Therefore, withaferin A and withaferin A containing plant extracts might be used for the alleviating mitochondrial toxins induced parkinsonism in human.

Thus, the present study depicts the heavy metal bioaccumulation and reduction capability of in vitro shoots of *W. somnifera* which resulted in the in planta synthesis and accumulation of Ag and Pb NPs. Further, silver treated *in vitro* shoots (E1D3T6) found to be more potent in reversing PD pathological symptoms compared to other test samples. Thus, it can be used for therapeutic purposes.